Ketoconazole Suppresses Prostaglandin E_2-Induced Cyclooxygenase-2 Expression in Human Epidermoid Carcinoma A-431 Cells

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Cyclooxygenase-2 is a key enzyme in the conversion of arachidonic acid to prostaglandins. The overexpression of cyclooxygenase-2 has been reported in skin cancer cells, and may be involved in carcinogenesis. Prostaglandin E_2, the end product of cyclooxygenase-2-induced catalysis, autoamplifies the cyclooxygenase-2 expression. It is suggested that an anti-mycotic drug, ketoconazole may inhibit carcinogenesis. We herein investigated if ketoconazole may inhibit prostaglandin E_2-induced cyclooxygenase-2 expression in human epidermoid carcinoma A-431 cells. Ketoconazole suppressed prostaglandin E_2-induced cyclooxygenase-2 protein and mRNA expression and promoter activation in A-431; the suppressive effects of ketoconazole were counteracted by cyclic adenosine monophosphate analog. Analyses using deleted or mutated cyclooxygenase-2 promoters revealed that cyclic adenosine monophosphate response element (− 59 to − 53 bp) on the promoter was involved in prostaglandin E_2-induced stimulation and ketoconazole-induced inhibition of the promoter activity. Electrophoretic mobility shift assays indicated that cyclic adenosine monophosphate response element binding protein and activating transcription factor-1 may constitutively bind to cyclic adenosine monophosphate response element on cyclooxygenase-2 promoter. Prostaglandin E_2 increased the proportion of phosphorylated forms among total bound cyclic adenosine monophosphate response element binding protein/activating transcription factor-1, and the effect was suppressed by ketoconazole. Prostaglandin E_2 induced the phosphorylation of cyclic adenosine monophosphate response element binding protein and activating transcription factor-1, and the phosphorylation was suppressed by cyclic adenosine monophosphate-dependent protein kinase (protein kinase A) inhibitor, indicating protein kinase A-mediated phosphorylation. Ketoconazole suppressed the prostaglandin E_2-induced phosphorylation of cyclic adenosine monophosphate response element binding protein/activating transcription factor-1. Prostaglandin E_2 increased intracellular cyclic adenosine monophosphate level by activating adenylate cyclase in A-431, and the increase was suppressed by ketoconazole. These results suggest that ketoconazole may suppress prostaglandin E_2-induced cyclooxygenase-2 expression by inhibiting the cyclic adenosine monophosphate signal in A-431, and stress its anti-cancer effect. ***Key words: activating transcription factor-1/cyclic adenosine monophosphate response element binding protein/phosphorylation. J Invest Dermatol 119:174–181, 2002***

It is reported that prostaglandin (PG) E_2 is involved in the progression of skin cancers; PGE_2 induces cancer cell proliferation, inhibits apoptosis (Tsuji and DuBois, 1995), promotes angiogenesis (Gallo *et al*, 2001), and inhibits immune surveillance in cancers of several tissues, including skin (Sheng *et al*, 1998; Higashi *et al*, 2000). PGE_2 synthesis is enhanced in skin cancers (Muller-Decker *et al*, 1995; Kagoura *et al*, 2001). The synthesis of prostaglandins is dependent on the activity of cyclooxygenase (COX), which converts arachidonic acid to PGH_2, the common precursor to all prostaglandins. There are two isoforms of COX: COX-1 and COX-2. COX-1 is constitutively expressed in most cell types, whereas COX-2 expression is basally at low level in most tissues, but is induced by phorbol esters, bacterial endotoxins, or growth factors (Tang *et al*, 2001). It is reported that COX-2 expression is enhanced in skin cancers, especially squamous cell carcinoma (Muller-Decker *et al*, 1999; Gallo *et al*, 2001), which is considered to be the cause of PGE_2 overproduction (Muller-Decker *et al*, 1995). A recent study reported that the suppression of COX-2 expression led to inhibition of the growth of human skin cancer (Higashi *et al*, 2000). It is thus a key therapeutic target for skin cancer to prevent COX-2 expression.

It is reported that PGE_2, the end product of COX-2, promotes the expression of its own synthesizing enzyme COX-2 in human airways smooth muscle cells (Bonazzi *et al*, 2000), human monocytes (Hinz *et al*, 2000), murine keratinocytes (Maldve *et al*,

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Abbreviations: AC, adenylate cyclase; ATF-1, activating transcription factor-1; Bt_2, dibutyryl; cAMP, 3′, 5′-adenosine cyclic monophosphate; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, cAMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; H-89, *N*-[2-((*p*-bromocinnamyl)aminoethyl)-5-isoquinolinesulfonamide; ketoconazole, ketoconazole; NF-κB, nuclear factor κB; PKA, protein kinase A.

2000), and especially in human prostate, breast, and colon carcinoma (Tjandrawinata *et al*, 1997). Such autoregulatory action of PGE_2 may be mainly mediated by inducing 3′,5′-adenosine cyclic monophosphate (cAMP) signal as cell surface PGE_2 receptors, EP_2 and EP_4, are positively coupled to adenylate cyclase (AC), which synthesizes cAMP (Negishi *et al*, 1993; Hinz *et al*, 2000). The other cAMP-elevating agents, such as forskolin or adenosine A_{2a} receptor agonist also promoted COX-2 expression in rat microglia (Fiebich *et al*, 1996), which also supports the cAMP-induced COX-2 expression. The human COX-2 promoter contains cAMP response element (CRE) (− 59 to − 53 bp relative to the transcriptional start site) (Inoue *et al*, 1994), and this element may be involved in PGE_2-induced COX-2 gene expression (Hinz *et al*, 2000). As COX-2 mRNA is highly degradable and unstable due to the AU-rich sequences in 3′-untranslated region (Ristimaki *et al*, 1994), the autoamplification of COX-2 expression by PGE_2 may sustain the enzyme levels in human prostate, breast, and colon carcinoma (Tjandrawinata *et al*, 1997). To suppress this autoamplification by PGE_2 may therefore lead to the inhibition on the growth of cancer cells.

Recently it has been reported that an anti-mycotic ketoconazole is therapeutically effective for cancers in several tissues, including skin (Trachtenberg and Pont, 1984; Janssen *et al*, 1989). Ketoconazole inhibited cytochrome P450-dependent enzyme 11-hydroxylase (Loose *et al*, 1983), which suppressed testosterone production and resulted in the inhibition on the growth of testosterone-dependent prostate cancer (Trachtenberg and Pont, 1984). Ketoconazole also inhibits the activity of P450-dependent aryl-hydrocarbon-hydroxylase or 7-ethoxyresorufin-deethylase involved in the synthesis of carcinogens and thus reduces the skin carcinogenic potential (Janssen *et al*, 1989). Ketoconazole also inhibits the activity of the other enzymes such as AC (Stalla *et al*, 1988), 5-lipoxygenase (Bettens *et al*, 1986), or calmodulin-dependent enzymes (Wolff *et al*, 1993). A recent study reported that ketoconazole inhibited arachidonic acid-induced COX-2 expression in rat intestinal epithelial cells by inhibiting the monoxygenase-catalyzed synthesis of 14,15-epoxyeicosatrienoic acid, the direct inducer of COX-2 expression, from arachidonic acid (Peri *et al*, 1998); however, it is unknown whether ketoconazole may alter PGE_2-induced COX-2 expression.

In this study, we investigated if ketoconazole may suppress PGE_2-induced COX-2 expression in human epidermoid carcinoma A-431 cells. We have obtained the inhibitory effects of ketoconazole. We further analyzed the *cis*-elements on COX-2 promoter and the transcription factors responsible for the PGE_2-induced stimulation and ketoconazole-induced inhibition.

MATERIALS AND METHODS

Reagents Lipopolysaccharide from *Escherichia coli* (serotype 025:B6) was obtained from Sigma (St Louis MO). Dibutyryl cAMP (Bt_2cAMP), *N*-[2-((*p*-bromocinnamyl)aminoethyl)-5-isoquinolinesulfonamide (H-89), and 3-isobutyl-1-methylxanthine were obtained from Calbiochem (La Jolla, CA). Ketoconazole was from Janssen Pharmaceutica N.V. (Beerse, Belgium). Anti-c-Jun antibody, anti-COX-1 antibody, anti-COX-2 antibody, and anti-CCAAT/enhancer binding protein (C/EBP) δ antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cAMP response element binding protein (CREB) antibody, anti-activating transcription factor-1 (ATF-1) antibody, and anti-phospho-CREB/ATF-1 antibody recognizing both phosphorylated CREB and ATF-1 were from New England Biolabs (Beverly, MA).

Cell line and culture conditions Human epidermoid carcinoma A-431 cells were purchased from Dainippon Pharmaceutical (Osaka, Japan), and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U penicillin per ml, and 100 μg streptomycin per ml.

Plasmids and transfections The phPES2 series of firefly luciferase constructs containing wild-type or mutated human COX-2 promoter fragments were prepared and cloned into pGL3 basic vector (Promega, Madison, WI) as described (Inoue *et al*, 1994, 1995; Inoue and Tanabe, 1998). p4x nuclear factor κB (NF-κB)-SV-luc, p4xC/EBP-SV-luc, and p4xCRE-SV-luc were constructed by inserting four copies of NF-κB sequences (5′-GTGGGGACTACCCCCT-3′), C/EBP sequences (5′-GGCTTACGCAATTTT-3′), and CRE sequences (5′-CATTTCGTC-ACATGG-3′) on COX-2 promoter, respectively, in front of minimal SV40 promoter upstream of firefly luciferase reporter as described (Aronica *et al*, 1994; Maehara *et al*, 2000). Transient transfections were performed using Lipofectamine Plus reagent (Gibco/BRL) according to the manufacturer's protocol. Briefly, A-431 cells (2×10^5) were plated in six-well plates the day before transfection and grown to about 90% confluence. 1.8 μg of luciferase construct and 0.2 μg of Rous sarcoma virus β-galactosidase vector per well was prepared in 100 μl serum-free and antibiotic-free DMEM and incubated with 3 μl of Lipofectamine Plus reagent at room temperature for 15 min, followed by addition of 2.5 μl of lipofectamine. After incubation at room temperature for another 15 min, the mixture was added into wells containing 0.8 ml DMEM. Transfections were allowed to proceed for 5 h and cells were washed and incubated in serum-free DMEM for 18 h. Cells were washed, and pretreated with serum-free DMEM alone or with 1 μM ketoconazole in the presence or absence of 100 μM Bt_2cAMP for 30 min, then treated with 1 μM PGE_2 for 4 h in the presence or absence of the above-mentioned reagents. In some experiments, cells were also treated with 1 μg lipopolysaccharide per ml during the same period. Cell extracts were prepared and luciferase activities were quantified using luciferase assay system (Promega). The same cell extracts were assayed for β-galactosidase activity using chemiluminescent Galacto-Light kit (Tropix, Bedford, MA). All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbach, Germany). The results obtained in each transfection were normalized for β-galactosidase activity and expressed as relative luciferase activity.

Western blot analysis A-431 cells (4×10^5) were seeded on a 6 cm Petri dish. When subconfluent, the cells were washed and incubated in 2 ml of serum-free DMEM for 24 h. The medium was then removed, and cells were incubated in 1.5 ml of serum-free DMEM for a further 4 h. The cells were pretreated with ketoconazole at indicated concentrations in the presence or absence of 100 μM Bt_2cAMP for 30 min, then treated with 1 μM PGE_2 in the presence or absence of the above-mentioned reagents for 4 h. Cells were then lyzed in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 50 μg aprotinin per ml, 50 μg leupeptin per ml, and centrifuged at 10 000 g for 10 min. Protein concentration in the supernatant was determined by Bio-Rad DC reagent (Bio-Rad Laboratories, Hercules, CA). For Western analysis, 20 μg of proteins were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel. The proteins were transferred to a PVDF membrane by electroblotting. The membrane was then blocked in 5% nonfat dry milk TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) at room temperature for 1 h. Primary antibody was diluted at 1:1000 in 5% bovine serum albumin/TBST for antibodies against phospho-CREB/ATF-1, CREB, or ATF-1 or at 1:4000 for anti-COX-2 and anti-COX-1 antibodies. The membrane was incubated at room temperature for 2–3 h and washed 3×5 min with TBST, then developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Quantification was performed by scanning the blot into Photoshop Adobe and performing densitometry with NIH Image Software.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) A-431 cells were incubated as above for 2 h, and total cellular RNA was extracted using mRNA purification kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. cDNA was made from RNA samples as described (Tjandrawinata *et al*, 1997). Primer sequences for COX-2 and for the internal control β-actin as well as PCR conditions are as described (Tjandrawinata *et al*, 1997). The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide, viewed by ultraviolet light, and photographed. Densitometric analysis was performed by scanning the bands into Photoshop and performing densitometry with NIH Image Software. Results are expressed for COX-2 product as the ratio relative to β-actin product.

Electrophoretic mobility shift assays The following sequences derived from the human COX-2 promoter containing CRE were used: PE2CRE1, 5′-GAAACACTCATTTCGTCACATGG-3′; PE2CRE2, 5′-CAAGCCCATGTGACGAAATGA-3′. They were annealed and 5′-overhangs were labeled by incorporation of [^{32}P]deoxycytidine triphosphate with Klenow DNA polymerase. For gel shift assays, 2–5 μg of nuclear protein extracts were incubated at room temperature for

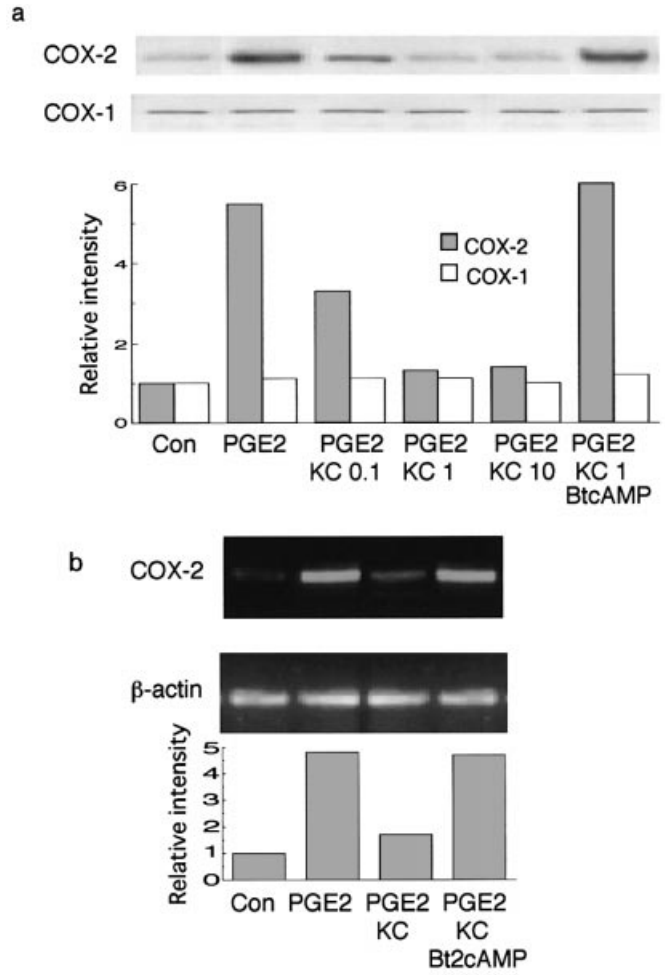

Figure 1. PGE_2-induced COX-2 protein and mRNA expression, and the ketoconazole-mediated inhibition on the expression. (*a*) A-431 cells were preincubated for 30 min with medium alone or with 0.1, 1, or 10 μM ketoconazole in the presence or absence of 100 μM Bt_2cAMP, and then incubated with or without 1 μM PGE_2 in the presence or absence of above-mentioned reagents for another 4 h. Cells were harvested, and proteins were extracted and analyzed by Western blotting. The lower graph is the quantitation of each signal in the upper immunoblot. Values are signal intensity relative to that in untreated cells (control). (*b*) The cells were preincubated with medium alone or with 1 μM ketoconazole in the presence or absence of 100 μM Bt_2cAMP for 30 min, then incubated with or without 1 μM PGE_2 in the presence or absence of above-mentioned reagents for 2 h. RNA was isolated, and reverse transcription–PCR was performed. The intensity of the band for COX-2 was corrected to that for β-actin. The lower graph shows the corrected intensities relative to those in untreated cells (set as 1.0). The results shown are representative of four separate experiments.

5 min with a mixture containing 6 mM HEPES (pH 7.9), 0.4 mM ethylenediamine tetraacetic acid, 125 mM KCl, 10% glycerol, 0.05 μg poly dI-dC per μl, 1 mM dithiothreitol, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 50 μg aprotinin per ml, 50 μg leupeptin per ml. Approximately, 1 ng labeled probe was added and the reactions were incubated at room temperature for another 20 min. In antibody supershift experiments, the nuclear extracts were preincubated with various amounts of antibodies on ice for 30 min before the addition of probe. Reactions were then fractionated on a nondenaturing 5% polyacrylamide gel in 0.5 × TBE (25 mM Tris–HCl, 0.5 mM ethylenediamine tetra-acetic acid, 25 mM boric acid). The gels were dried and visualized with phosphorimager (Molecular Dynamics, Sunnyvale, CA).

PGE_2 production A modification of the method of Yanagi and Komatsu (1976) was used. Briefly, 1×10^5 cells per well of A-431 were plated in six-well plates and grown to 60% confluence in growth medium. The cells were washed and serum-deprived as described above. The cells were pretreated with serum-free DMEM alone or with 1 μM ketoconazole for 30 min, then treated with 1 μM PGE_2 in the presence or absence of ketoconazole for 4 h. The cells were harvested and suspended in 20 mM Tris–HCl (pH 7.4), containing 5 mM tryptophan and sonicated. The sonicated cells were incubated for 2 min with 5 μM [^{14}C]arachidonic acid in 100 mM Tris–HCl (pH 8.0), 2 μM hematin, and 5 mM tryptophan. After adding n-hexane ethyl acetate and centrifuging the preparation at 1000 g for 10 min, the amount of synthesized [^{14}C]PGE_2 in the aqueous phase was determined by measuring radioactivity. Production of PGE_2 was normalized to protein concentrations.

Measurement of cAMP A-431 cells were cultured under the indicated conditions, and were harvested and lyzed with ethanol. The cell lysates were centrifuged and the supernatants were dried under vacuum. The dried samples were dissolved in acetate buffer (pH 5.8), and cellular cAMP contents were measured with enzyme-linked immunosorbent assay (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The sensitivity of the assay was 12 fmol per assay well. The cellular cAMP level was presented as pmol/10^6 cells.

Assays of cAMP-dependent protein kinase (protein kinase A; PKA) A-431 cells were cultured under the indicated conditions, and were lyzed in the buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid, 1 μg aprotinin per ml, 1 μg pepstatin per ml, 1 μg leupeptin per ml, 15 mM benzamidine, and 3.75 mM β-mercaptoethanol. The cell lysate was assayed for the activity of PKA using an enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan) by examining the phosphorylation of plate-bound peptide substrate in the presence or absence of 2 μM cAMP for 10 min at room temperature. The plates were sequentially incubated with biotinylated antibody to the phosphorylated substrate, peroxidase-conjugated streptavidin, *o*-phenylenediamine, then the reaction was stopped, and optical density at 492 nm was read. The net optical density was calculated by subtracting the density in the presence of 1 μM of specific PKA inhibitor KT-5720 (Calbiochem) from the total density. The PKA activity was expressed as an activity ratio, which is defined as the net optical density in the absence of exogenous cAMP divided by the net optical density in the presence of cAMP.

Measurement of AC activity The lysate of A-431 cells described above was centrifuged at 23,600 × *g* for 10 min. The pellet was used as a particulate fraction for AC assays as described (Salomon *et al*, 1974; Choi *et al*, 1992). The AC activity of the particulate fraction was measured at 37°C for 10 min in 20 mM Tris–HCl (pH 7.4), 1 mM [α-^{32}P]adenosine triphosphate (30 Ci per mmol) (Amersham), 1 mM [^{3}H]cAMP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM $MgCl_2$, 0.2 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 20 mM creatine phosphate, and 100 units per ml creatine phosphokinase. AC activity was presented as pmol cAMP formed per min per mg protein.

Statistical analyses One-way analysis of variance with Scheffe's multiple comparison test was used for the data in **Figs 2–4 and 7**.

RESULTS

The effects of ketoconazole on PGE_2-induced COX-2 protein and mRNA expression COX-2 protein was constitutively expressed in A-431 cells, and the expression was enhanced by PGE_2 (**Fig 1*a***). The PGE_2-induced enhancement of COX-2 protein level was inhibited by ketoconazole in a concentration-dependent manner with maximal inhibition at 1 μM, and the inhibition was counteracted by cAMP analog, Bt_2cAMP, indicating the involvement of cAMP in the ketoconazole-induced inhibition. COX-1 protein expression was not altered by PGE_2, PGE_2 + ketoconazole, or PGE_2 + ketoconazole + Bt_2cAMP. As analyzed by reverse transcription–PCR, the steady-state mRNA level for COX-2 was enhanced by PGE_2, and the enhancement was partially suppressed by ketoconazole, which was also counteracted by Bt_2cAMP (**Fig 1*b***).

We then analyzed if the enhancement or inhibition of COX-2 expression may result in the increase or decrease of PGE_2 production in A-431 cells, respectively. The incubation with

PGE_2 for 4 h increased PGE_2 synthetic activity 4.1-fold of controls, which was reduced by the coincubation with ketoconazole by 75% (**Fig 2**); however, ketoconazole *per se* did not suppress PGE_2 synthetic activity when added to the reaction mixture. Thus the PGE_2-induced stimulation and ketoconazole-induced inhibition of COX-2 expression resulted in the increase and decrease of PGE_2 synthetic activity in A-431 cells, respectively.

The effects of ketoconazole on PGE_2-induced COX-2 promoter activation We then analyzed if ketoconazole may suppress PGE_2-induced upregulation of COX-2 promoter activity. The activity of COX-2 promoter (–327 to +59) was increased by PGE_2 4.8-fold of controls, and the increase was suppressed by ketoconazole, which was counteracted by Bt_2cAMP (**Fig 3*b***, the left four columns). These indicate that PGE_2-responsive and ketoconazole-sensitive *cis*-elements may be located within the region from –327 to +59, and that cAMP may be involved in the ketoconazole-induced suppression of the promoter activity. Constructs with deletions were then used to analyze the PGE_2-responsive and ketoconazole-sensitive elements. Deletion of the sequence up to –124 did not significantly affect the induction by PGE_2 and suppression by ketoconazole on the promoter activity, although the deletion from –220 to –124 reduced the basal promoter activity by 30%, indicating that the sequences between –220 and –124 may partially confer the basal promoter activity. As the NF-κB element is located within the region –327 to –220, and the C/EBP element is located within the region –220 and –124 (**Fig 3*a***), the NF-κB and C/EBP sites may not be essential for the induction by PGE_2 and suppression by ketoconazole on the COX-2 promoter activity, but C/EBP may be related to the basal promoter activity. Deletion up to –52 dramatically decreased the basal promoter activity, and abrogated the induction by PGE_2 and the suppression by ketoconazole on the promoter activity. This indicates that the sequences between –124 and –52, containing CRE may be essential for basal promoter activity and PGE_2-induced promoter activity and also for the ketoconazole-induced suppression. This proposition was further supported by constructs containing site-directed mutations. The mutation of either NF-κB or C/EBP site did not affect the PGE_2-induced stimulation and ketoconazole-induced suppression of COX-2 promoter activity, although the latter mutation partially reduced (by 27%) the basal promoter activity (**Fig 3*c***). In contrast, the mutation of CRE site

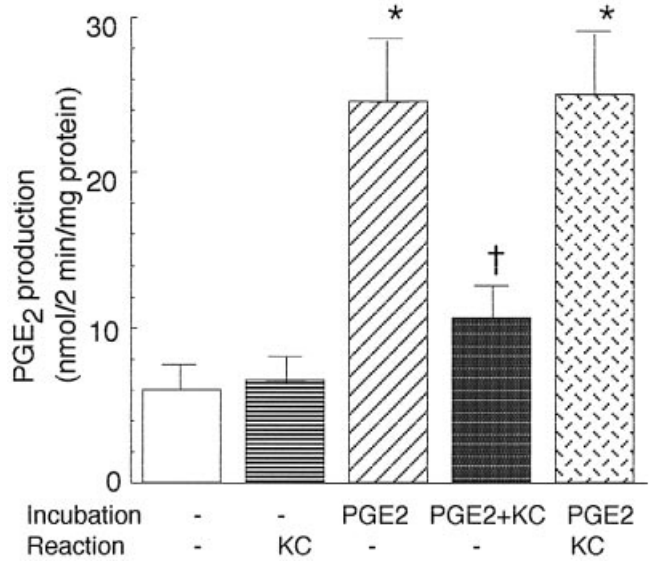

Figure 2. PGE_2 synthetic activity in PGE_2-treated A-431 cells and the effects of ketoconazole on the activity. A-431 cells were preincubated with medium alone or with ketoconazole 1 μM for 30 min, then incubated with or without 1 μM PGE_2 in the presence or absence of ketoconazole for another 4 h. The cells were harvested, sonicated, and the cell sonicate was incubated with 10 μM [^{14}C]arachidonic acid for 2 min in the presence or absence of ketoconazole 1 μM. The synthesis of [^{14}C]PGE_2 was measured and normalized to protein concentrations. The data are mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control values of cells with medium alone, and †$p < 0.05$ *vs* values with PGE_2 alone, by one-way analysis of variance with Scheffe's multiple comparison test.

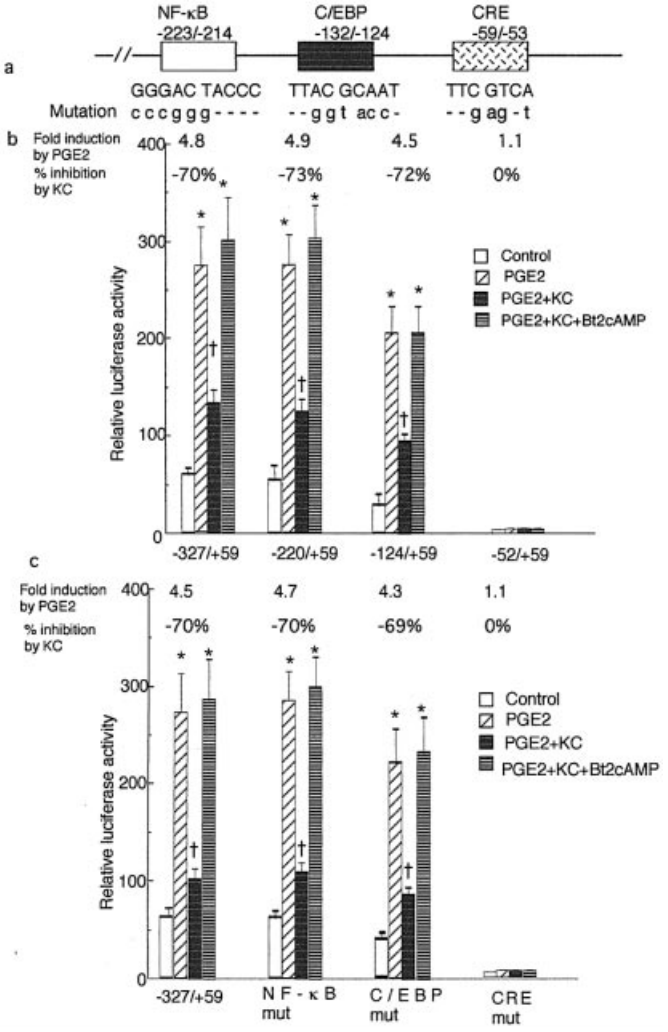

Figure 3. The PGE_2-induced stimulation of COX-2 promoter and the effects of ketoconazole on the stimulation. (*a*) Schematic representation of human COX-2 promoter. The locations of NF-κB, C/EBP, and CRE are shown with their sequences, and the lower case letters represent the mutations. The nucleotide positions are relative to the transcriptional start site. (*b*) A-431 cells were transiently transfected with 1.8 μg of a series of human COX-2 promoter deletion constructs ligated to luciferase (–327/+59, –220/+59, –124/+59) and 0.2 μg β-galactosidase vector. (*c*) A-431 cells were transfected with wild-type COX-2 promoter luciferase construct (–327/+59) or mutant construct derived from –327/+59, and β-galactosidase vector. The mutations exist at NF-κB, C/EBP, or CRE. After transfection, cells were allowed to recover for 18 h and pretreated with medium alone or with 1 μM ketoconazole in the presence or absence of Bt_2cAMP 100 μM for 30 min, then treated with or without 1 μM PGE_2 in the presence or absence of the agents above for 4 h. Cells were harvested and luciferase and β-galactosidase activities were measured. Relative luciferase activities normalized to β-galactosidase activities were shown. The data are mean ± SEM of four separate experiments. The fold induction by PGE_2 *vs* basal promoter activity and percentage inhibition by ketoconazole on the PGE_2-mediated induction are shown for each promoter. *$p < 0.05$ *vs* control values, and †$p < 0.05$ *vs* values with PGE_2 alone, by one-way analysis of variance with Scheffe's multiple comparison test.

decreased the basal promoter activity by 80%, and abrogated the induction by PGE_2 and suppression by ketoconazole. These results suggest that CRE on COX-2 promoter may be essential for the basal activity. The CRE may also be the PGE_2-responsive and ketoconazole-sensitive element, although it is also possible that the mutation of the basal activity-conferring element, CRE may prevent the occurrence of any transcriptional activation either through CRE or the other elements.

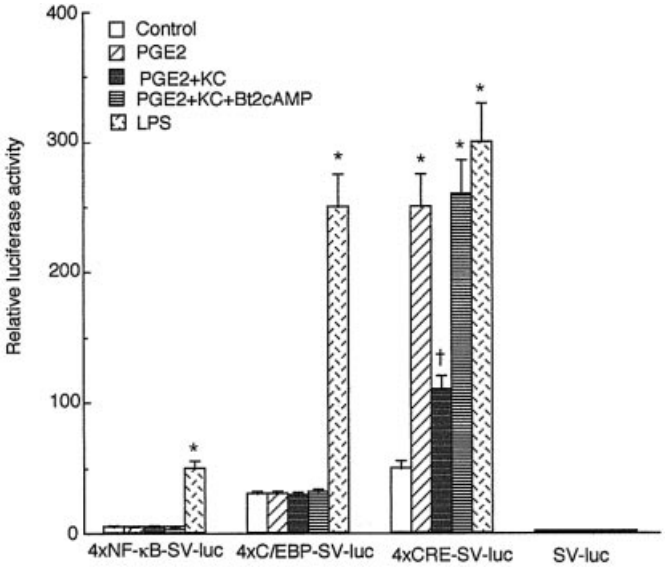

Figure 4. PGE_2-induced activation and ketoconazole-induced inhibition on the enhancer activities of NF-κB, C/EBP, and CRE. A-431 cells were transiently transfected with luciferase reporter plasmids driven by NF-κB, C/EBP, or CRE together with β-galactosidase vector. The cells were allowed to recover for 18 h and preincubated with medium alone or with 1 μM ketoconazole in the presence or absence of 100 μM Bt_2cAMP for 30 min, then incubated with or without 1 μM PGE_2 in the presence or absence of reagents above for 4 h. In parallel, the cells were also incubated with 1 μg lipopolysaccharide per ml as a positive control during the same period. The cells were then harvested and luciferase and β-galactosidase activities were assayed. The results are shown as relative luciferase activities normalized for β-galactosidase activities, and represent mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control values, and †$p < 0.05$ *vs* values with PGE_2 alone, by one-way analysis of variance with Scheffe's multiple comparison test.

To determine if ketoconazole may inhibit PGE_2-induced activation of CRE-dependent gene transcription, A-431 cells were transfected with plasmid containing four repeats of CRE in front of the enhancerless minimal SV40 promoter and luciferase reporter, and were incubated with PGE_2 in the presence or absence of ketoconazole. The enhancer activity of CRE was evaluated by the relative luciferase activity in the lysates of the transfected cells. The enhancerless SV40 promoter–luciferase vector produced very low background luciferase activity, which was not altered by PGE_2 or PGE_2 + ketoconazole (**Fig 4**). The enhancer activity of CRE was constitutively high, which was increased by PGE_2 5.1-fold (**Fig 4**). Ketoconazole inhibited the PGE_2-induced increase of CRE enhancer activity by 70%, and the inhibition was counteracted by Bt_2cAMP. The enhancer activity of NF-κB was constitutively low, which was not altered by PGE_2, PGE_2 + ketoconazole, or PGE_2 + ketoconazole + Bt_2cAMP. The enhancer activity of C/EBP was not altered by these agents, either. The enhancer activities of CRE, NF-κB, and C/EBP were promoted by a positive control lipopolysaccharide, which is known to enhance CRE, NF-κB, and C/EBP-mediated COX-2 gene transcription (Inoue and Tanabe, 1998; Caivano *et al*, 2001). These results suggest that ketoconazole may inhibit PGE_2–induced COX-2 transcription through CRE, and that cAMP may be involved in the inhibitory effect of ketoconazole.

The transcription factors binding to COX-2 CRE To identify transcription factors that bind to the COX-2 CRE, electrophoresis mobility shift assays were performed. Incubation of A-431 nuclear extracts with the COX-2 CRE probe produced the DNA–protein complex band (**Fig 5*a,b***), which was competed by excess cold COX-2 CRE (**Fig 5*b***). The binding activity of A-431 nuclear extracts to the COX-2 CRE did not change upon the treatment with PGE_2 alone or PGE_2 + ketoconazole (**Fig 5*a,b***). Anti-CREB antibody potently and anti-ATF-1 antibody moderately supershifted the band (**Fig 5*a***), indicating that this band contains CREB and ATF-1. Though it is reported that c-Jun transcription factor (Hsue and Lai, 1995) or C/EBPδ (Inoue *et al*, 1995) also binds to COX-2 CRE, neither anti-c-Jun nor anti-C/EBPδ antibody supershifted or cleared the band (**Fig 5*b***), indicating the absence of c-Jun or C/EBPδ in the band. As the phosphorylation of CREB and ATF-1 enhances their transcriptional activity (Tang *et al*, 2001), it was examined whether the phosphorylated CREB or ATF-1 in PGE_2-treated

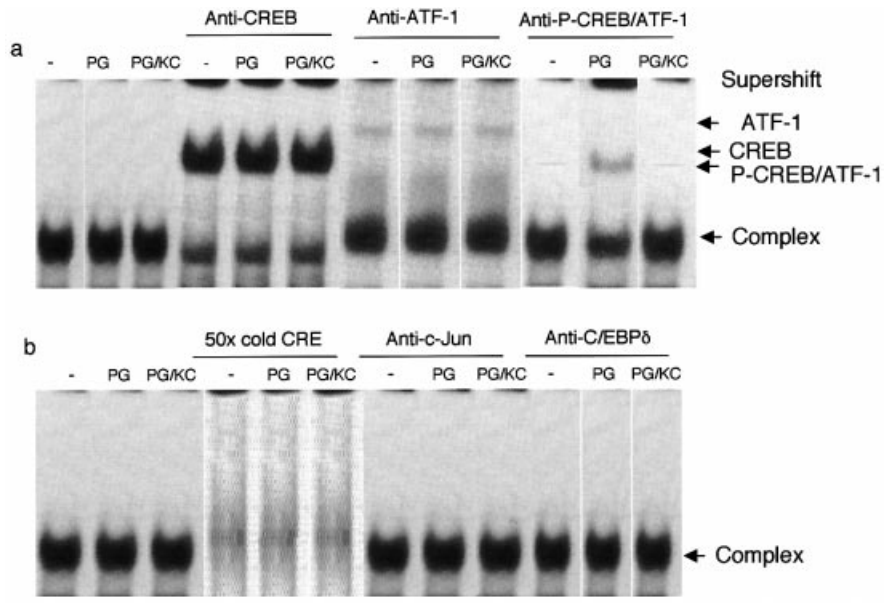

Figure 5. Electrophoretic mobility shift assay targeting the CRE site. A-431 cells were preincubated with medium alone or with 1 μM ketoconazole for 30 min, then incubated with or without 1 μM PGE_2 in the presence or absence of ketoconazole for 2 h, and nuclear extracts were prepared. The nuclear extracts were incubated with ^{32}P-labeled oligonucleotides containing COX-2 CRE site. Cold chase experiments were performed with a 50-fold excess of a competitor oligonucleotide (*b*). In supershift assays, antibodies for CREB, ATF-1, phospho-CREB/ATF-1 (*a*) or for c-Jun, C/EBPδ (*b*) were incubated for 30 min before the addition of the probe. *Arrows* indicate the DNA–protein complexes and supershifted complexes.

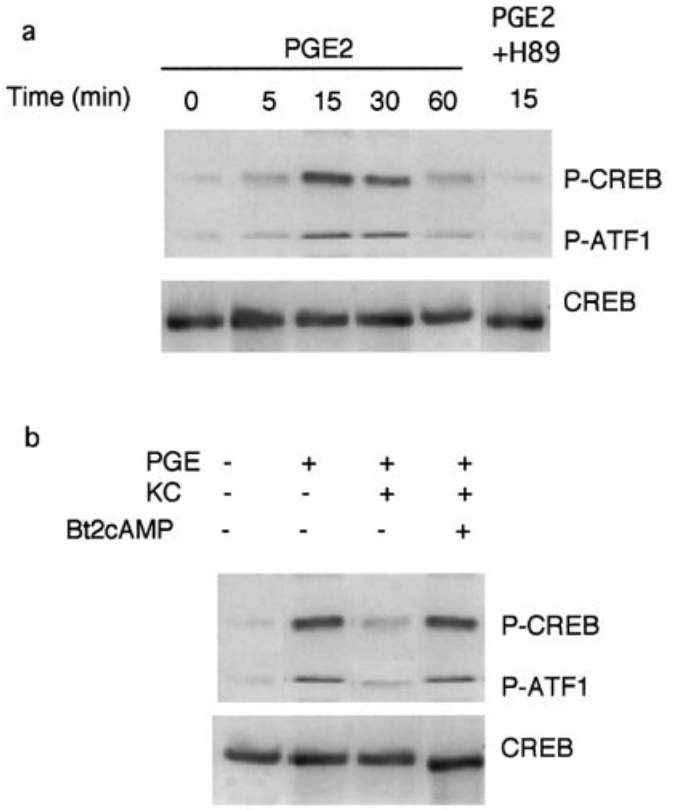

Figure 6. The PGE_2-induced phosphorylation of CREB/ATF-1 and ketoconazole-induced inhibition of the phosphorylation. (*a*) A-431 cells were preincubated with medium alone or with 0.1 μM H-89 for 30 min, then incubated with 1 μM PGE_2 during the indicated periods. Twenty micrograms of whole cell lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel and probed with an antibody recognizing both phospho-CREB and phospho-ATF-1. A parallel blot was probed with an antibody for total CREB. (*b*) A-431 cells were preincubated with medium alone or with 1 μM ketoconazole in the presence or absence of 100 μM Bt_2cAMP for 30 min, then incubated with or without 1 μM PGE_2 for 15 min The phosphorylation of CREB/ATF-1 in the cell lysates was analyzed as described above. The results are representative of four separate experiments.

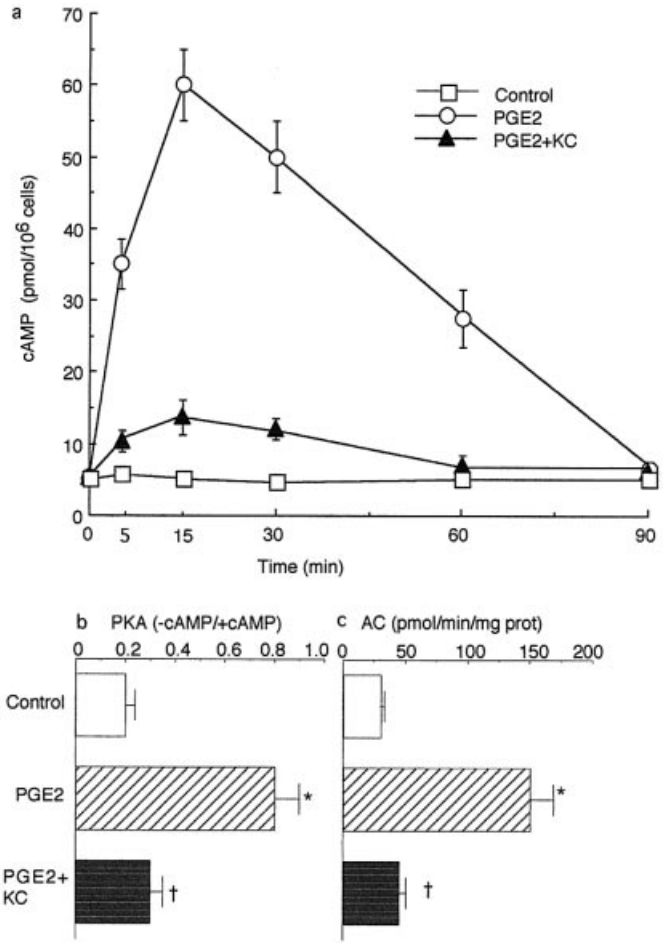

Figure 7. The effects of ketoconazole on cAMP level, PKA, and AC activities in PGE_2-treated A-431 cells. A-431 cells were preincubated with medium alone or with 1 μM ketoconazole for 30 min, then incubated with or without 1 μM PGE_2 in the presence or absence of ketoconazole. The cells were harvested and assayed for cAMP level during the indicated periods (*a*). PKA activity in the cell lysate (*b*) or AC activity in the particulate fraction (*c*)was analyzed after 15 min of incubation. The data are mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control values, and †$p < 0.05$ *vs* values with PGE_2 alone, by one-way analysis of variance with Scheffe's multiple comparison test.

samples may also bind to the COX-2 CRE, using the antibody recognizing phosphorylated forms of both CREB and ATF-1. The band supershifted by anti-phospho-CREB/ATF-1 is more obvious in PGE_2-treated samples than in control untreated samples, and the supershift was reduced by ketoconazole treatment (**Fig 5*a***). These results indicate that CREB/ATF-1 may constitutively bind to CRE, and that PGE_2 may increase the proportion of phosphorylated forms among total bound CREB/ATF-1 proteins, which may enhance the transcription through CRE. Ketoconazole may suppress the PGE_2-mediated increase of the phosphorylated CREB/ATF-1 bound to CRE.

The effects of ketoconazole on PGE_2-induced phosphorylation of CREB/ATF-1 We examined if CREB and ATF-1 may be phosphorylated by PGE_2 treatment. PGE_2 induced the phosphorylation of CREB and ATF-1 as early as 5 min after the treatment, and the phosphorylation was maximized at 15 min and lasted about 1 h (**Fig 6*a***). The PGE_2-induced phosphorylation was suppressed by cAMP-dependent protein kinase (PKA) inhibitor H-89, indicating PKA-mediated phosphorylation. The total level of CREB was not affected by PGE_2 or H-89 treatment. Ketoconazole suppressed the PGE_2-induced phosphorylation of CREB and ATF-1, and the suppression was counteracted by Bt_2cAMP (**Fig 6*b***). These results indicate that PGE_2 may induce the phosphorylation and resultant activation of CREB/ATF-1 via PKA, and that the phosphorylation may be suppressed by ketoconazole.

The effects of ketoconazole on PGE_2-induced cAMP signal and AC activity PGE_2 is known to increase intracellular cAMP level (Negishi *et al*, 1993), and cAMP appeared to be involved in the ketoconazole-mediated suppression of COX-2 induction by PGE_2. It was thus examined whether ketoconazole may alter the PGE_2-induced increase of the cAMP level. PGE_2 increased the cAMP level 9-fold of controls in A-431 cells 15 min after treatment, and the level gradually decreased and returned to the background at 90 min (**Fig 7*a***). Ketoconazole suppressed the PGE_2-induced increase of cAMP level by 85% at 15 min. The PGE_2-induced cAMP signal appeared to increase the PKA activity 4-fold of the controls (**Fig 7*b***), and the increase was also suppressed by ketoconazole by 75%, which paralleled the inhibition of CREB/ATF-1 phosphorylation (**Fig 6*b***). The incubation with PGE_2 increased the activity of cAMP-synthesizing enzyme AC 5-fold of controls in A-431 cells, and ketoconazole suppressed the increase of AC activity by 83% (**Fig 7*c***). These results suggest that ketoconazole may suppress PGE_2-induced cAMP signal by inhibiting AC activity. The ketoconazole-induced inhibition of

cAMP signal may lead to the inhibition of CREB/ATF-1 phosphorylation by PKA, and result in the suppression of PGE_2-induced COX-2 transcription in A-431 cells.

DISCUSSION

This study demonstrated that ketoconazole inhibited PGE_2-induced COX-2 expression in A-431 cells. PGE_2 induced cAMP signal in A-431 cells and thus enhanced the phosphorylation of CREB/ATF-1 by cAMP-dependent protein kinase (PKA), which led to the phospho-CREB/ATF-1-mediated COX-2 transcription. Ketoconazole suppressed the PGE_2-induced cAMP signal by inhibiting AC, which resulted in the inhibition of CREB/ATF-1 phosphorylation by PKA, and then led to the inhibition of COX-2 expression. Although the ketoconazole-induced suppression of AC has also been detected in human T cells (Kanda *et al*, 2001) or rat anterior pituitary cells (Stalla *et al*, 1988, 1989), this study directly shows that ketoconazole inhibits the phospho-CREB/ATF-1-mediated gene expression. The present results also indicate that ketoconazole may inhibit the phospho-CREB/ATF-1-mediated expression of the other genes related to carcinogenesis. As there exist the other transcription factors, such as activator protein-2, whose transcriptional activity is promoted by cAMP (Garcia *et al*, 1999), it should further be clarified if the cAMP-responsive transcription factors other than CREB/ATF-1 can also be the targets for ketoconazole.

It is reported that EP2 and EP_4 among four identified PGE_2 receptor isoforms are positively coupled to AC via guanine nucleotide-binding proteins (Maldve *et al*, 2000). Thus PGE_2-induced activation of AC may be mediated through either or both of EP_2 and EP_4 in A-431 cells. The mechanism for PGE_2-induced COX-2 expression is similar to that for an AC catalyst activator forskolin; forskolin enhanced PKA-mediated phosphorylation of CREB/ATF-1 by inducing cAMP signal and thus promoted COX-2 transcription in HaCaT cells (Tang *et al*, 2001). Another mechanism is also reported for cAMP-induced COX-2 transcription; cAMP-elevating agents, forskolin, luteinizing hormone, and follicle-stimulating hormone promoted C/EBPβ synthesis and enhanced C/EBPβ-mediated COX-2 transcription through C/EBP element in rat granulosa cells (Sirois and Richards, 1993). This mechanism is, however, unlikely for A-431 cells as PGE_2 did not enhance C/EBP-dependent transcription in these cells (**Fig 4**). It is also reported that PGE_2 enhanced C/EBPδ-mediated insulin-like growth factor-I gene expression through C/EBP element in rat osteoblasts (Umayahara *et al*, 1997). C/EBPδ binds to both C/EBP and CRE sites on human COX-2 promoter (Inoue *et al*, 1995), and C/EBPδ-mediated COX-2 transcription is enhanced in murine skin carcinoma cells (Kim and Fischer, 1998). C/EBPδ, however, may not be involved in PGE_2-induced COX-2 transcription in A-431 cells as C/EBPδ did not form a complex with the COX-2 CRE probe (**Fig 5*b***) and PGE_2 did not enhance C/EBP-dependent transcription in these cells (**Fig 4**).

Takahashi *et al* (1994) and Pilbeam *et al* (1995) also reported that $PGF_{2\alpha}$ promoted COX-2 expression by inducing phosphatidylinositol turnover in murine osteoblastic cell lines. The binding of $PGF_{2\alpha}$ to its receptor may activate phospholipase C, and may further promote the activity of downstream protein kinase C, which may enhance the activity of certain transcription factors, such as NF-κB or c-Jun to induce COX-2 expression. Among the receptors for PGE_2, EP_1 and several isoforms of EP_3 are coupled to phospholipase C (Pilbeam *et al*, 1995). Thus it is also possible that PGE_2 may induce COX-2 transcription via EP_1 or EP_3-mediated activation of phospholipase C/protein kinase C pathway. This mechanism is, however, unlikely for A-431 cells as $EP1/EP_3$ receptor agonist sulprostone did not induce COX-2 transcription in A-431 cells (unpublished observations). An alternative mechanism for PGE_2-induced COX-2 expression involves the peroxisome proliferator response element. Several prostaglandins are ligands for nuclear peroxisomal proliferator-activated receptors, and the ligand-bound peroxisomal proliferator-activated receptor heterodimerizes with nuclear retinoic acid X receptor, and the heterodimer binds to a peroxisome proliferator response element on certain genes, and induces the gene expression (Meade *et al*, 1999). 5′-flanking region of COX-2 gene contains the peroxisome proliferator response element at approximately 3900 bases upstream of the transcriptional start site, and peroxisome proliferator WY-14643 enhanced COX-2 transcription through this element in human mammary epithelial cells (Meade *et al*, 1999). Thus it should be examined if prostaglandins, including PGE_2, may also use this mechanism for COX-2 expression in A-431 cells.

Several studies reported that PGE_2 or other cAMP-elevating agents inhibited COX-2 expression; PKA inhibited the phosphorylation of c-Jun transcription factor by c-Jun NH_2-terminal kinase (Hsueh and Lai, 1995), which led to the inhibition of c-Jun-mediated COX-2 transcription in porcine aortic smooth muscle cells (Karim *et al*, 1997). In A-431 cells, however, such a manner of inhibition may not occur as c-Jun did not bind to CRE on COX-2 promoter in these cells (**Fig 5*b***). It is also reported that PKA inhibits the activity of extracellular signal-regulated kinase (Shapiro *et al*, 1996), which phosphorylates C/EBPβ and promotes the C/EBPβ-mediated COX-2 transcription through C/EBP element in murine mast cells (Reddy *et al*, 2000). Thus it is possible that PGE_2 may suppress the C/EBPβ-mediated COX-2 transcription by activating PKA. This possibility is also unlikely for A-431 cells as PGE_2 did not suppress C/EBP-dependent COX-2 transcription in these cells (**Fig 4**). It appears that PGE_2 may regulate COX-2 expression in a cell-type-specific and species-specific manner. The upregulation or downregulation of COX-2 expression by PGE_2 and the mechanism for the regulation may depend on the amounts and activities of the relevant transcriptional activators, repressors, and cofactors, and also on the activities of signaling molecules regulating these factors. At least in A-431 cells, PGE_2 enhanced phospho-CREB/ATF-1-mediated COX-2 expression via cAMP. It should further be elucidated whether PGE_2 may upregulate or downregulate COX-2 expression in the other skin cancer cells and also determine what the precise mechanisms for this regulation are. In this study, PGE_2 did not alter COX-1 expression (**Fig 1**) and the results are different from those in murine keratinocytes showing the enhancement of COX-1 expression by PGE_2 (Maldve *et al*, 2000). This may be due to the difference in the sequences of COX-1 gene between species and also due to the aberrant regulation associated with carcinogenesis; the PGE_2-mediated promotion of COX-1 expression may be suppressed in cancer cells by a specific undefined mechanism.

This study suggests that ketoconazole may inhibit PGE_2-driven positive feedback control of COX-2 expression. This effect of ketoconazole may prevent the sustained COX-2 expression and may lead to the inhibition on the growth of skin cancer. It is reported that COX-2-induced carcinogenesis consists of PGE_2-dependent and PGE_2-independent pathways (Higashi *et al*, 2000). COX possesses COX activity converting arachidonic acid to PGG_2, and peroxidase activity generating PGH_2, a direct precursor of PGE_2, from PGG_2. In addition to its role in PG synthesis, the peroxidase activity of COX contributes to superoxide production and subsequent alteration in intracellular redox status, which is associated with perturbations of cell growth and transformation (Higashi *et al*, 2000). Thus ketoconazole-induced inhibition of COX-2 expression may prevent both PGE_2-dependent and PGE_2-independent pathways for carcinogenesis. Thus ketoconazole may be more effective for the inhibition of cancer growth than the inhibitors of COX-2 catalytic activity, such as NS-398, which inhibits COX activity but not the peroxidase activity of COX-2. A recent study showed that COX-2 expression was detectable in premalignant lesions, such as actinic keratoses (Muller-Decker *et al*, 1999). Ketoconazole, therefore, may manifest chemopreventive effects to suppress the progression from such premalignant lesions into a more advanced status by inhibiting COX-2 expression. Previous studies revealed the anti-cancer effects of ketoconazole via

inhibition of cytochrome P450-dependent enzymes (Janssen *et al*, 1989). Taken together, ketoconazole may inhibit the multiple carcinogenic pathways dependent on cytochrome P450 and on AC.

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